Reduced Circulating Insulin-like Growth Factor I Levels Delay the Onset of Chemically and Genetically Induced Mammary Tumors

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ABSTRACT

Insulin-like growth factors (IGFs) play a crucial role in regulating cell proliferation and differentiation. The aim of this study was to examine the potential relationship between serum IGF-I levels and breast cancer risk. To do this, we studied liver-specific IGF-I gene-deleted (LID) mice, in which circulating IGF-I levels are 25% of that in control mice. Mammary tumors were induced in two ways: (*a*) by exposing mice to the carcinogen 7,12-dimethybenz (*a*)anthracene; and (*b*) by crossing LID mice with C3(1)/SV40 large T-antigen transgenic mice. In both models, LID mice exhibited a delayed latency period of mammary tumor development. In the 7,12-dimethybenz (*a*)anthracene-induced mammary tumor model, the incidence of palpable mammary tumors was significantly lower in LID mice (26% *versus* 56% in controls), and the onset of the tumors was delayed (74 ± 1.2 days in LID mice *versus* 59.5 ± 1.1 days in controls). Histological analysis showed extensive squamous metaplasia in late-stage mammary tumors of control mice, whereas late-stage tumors from LID mice exhibited extensive hyperplasia, but little metaplasia. In control mice, the onset of C3(1)/SV40- large T-antigen-induced mammary tumors occurred at 21.6 ± 1.8 weeks of age, whereas in LID mice the average age of onset was 30.2 ± 1.7 weeks. In addition, 60% of the mice in the control group developed two or more mammary tumors per mouse, whereas in the LID mice only 30% developed more than one mammary tumor per mouse. Our data demonstrate that circulating IGF-I levels play a significant role as a risk factor in the onset and development of mammary tumors in two well-established animal models of breast cancer.

INTRODUCTION

Tumor cells exhibit abnormal cellular activity, which is largely maintained and controlled by various peptide growth factors. Among these, the IGFs[2] play a crucial role in regulating cell proliferation and inhibiting apoptosis (1, 2). The IGFs are members of a well-characterized family of insulin-related peptides that includes insulin, IGF-I, and IGF-II. In the circulation, the IGFs bind to specific IGFBPs, of which six have been well characterized (3). The IGF-IR is a transmembrane tyrosine kinase receptor that mediates most of the effects of both IGF-I and IGF-II (4). The bioactivity of IGF-I in tissues is related to circulating IGF-I and IGFBP levels, as well as to local production of IGF-I, IGFBPs, and IGFBP-proteases. However, a direct causative relationship between circulating IGF-I and/or IGFBP levels, and the initiation or development of tumors has not yet been established. These observations have led investigators to ask whether circulating levels of IGF-I and IGFBPs may represent markers for the risk of tumor development. Increasingly, human epidemiological studies have shown a correlation between circulating levels of IGF-I and IGFBP-3, and the relative risk of developing colon, breast, prostate, and lung cancer (5–9).

Breast cancer cells respond to exogenous IGFs, and it has been proposed that breast cancer prognosis may be dependent on host-derived IGFs. It was demonstrated that birth weight and breast cancer risk in humans are positively correlated, which is consistent with the observation that low birth weight is associated with low IGF-I levels (10–12). In a pilot study that measured IGFBP levels in 80 breast tumor specimens, it was shown that all of the IGFBPs except IGFBP-1 were expressed in each of the samples examined (13). The expression of IGFBP-4 was correlated with the expression of markers of poor prognosis. In a broader study of 238 node-negative patients, it was shown that the IGFBP levels correlated with ER expression levels (14, 15). ER-positive tumors expressed high levels of IGFBP-4 and -5, whereas ER-negative tumors expressed high levels of IGFBP-3 (16). It was also shown that estrogen inhibits IGFBP-3 expression in human breast cancer cells, whereas antiestrogens can stimulate IGFBP-3 expression (16). Moreover, IGFBP-3 can be degraded by proteases such as cathepsin D, prostate-specific antigen, and plasmine, which are all detected in breast cancer cells (17–21). In general, it is believed that these proteases cleave IGFBP-3 and thereby release IGF-I from IGFBP-3, increasing IGF-I bioavailability.

Whereas it is tempting to hypothesize that endocrine-derived IGF-I could induce the initiation and progression of mammary cancer, there is currently no direct evidence to support this hypothesis. However, if endocrine IGF-I does affect tumor growth, we would expect IGF-I levels to be prognostic for the disease. Measurements of circulating IGF-I levels showed that breast cancer patients had significantly higher levels of circulating IGF-I than did normal patients, whereas there was no correlation between IGF-I levels and several other prognostic factors, such as ER and nodal status (22). A prospective study, which included 397 women with breast cancer and 620 age-matched controls, showed that among premenopausal women, those in the top tertile of IGF-I levels had a relative risk of breast cancer of 4.58 (95% confidence limits, 1.75–12.0), compared with those in the bottom tertile. However, no link was found between IGF-I levels and breast cancer in postmenopausal women (6). Although *in vitro* data predicts that IGF-I expression levels would be associated with a poor prognosis in breast cancer, the limited clinical data available does not support this hypothesis.

This study was designed to examine the potential relationship between plasma IGF-I levels and breast cancer risk in a mouse mammary tumor model. Using the Cre/*loxP* recombination system, we generated LID mice, in which circulating IGF-I levels are low (25% of that in control mice), whereas extrahepatic IGF-I gene expression is normal (23). Mammary tumors were induced using two models: (*a*) the carcinogen DMBA; and (*b*) C3(1)/SV40 LTA transgenic mice, in which 100% of the females develop mammary tumors (24). We show that the latency and incidence of mammary tumors induced by DMBA and by C3(1)/SV40-LTA are lower in LID mice, as compared with controls.

A Serum IGF-I levels
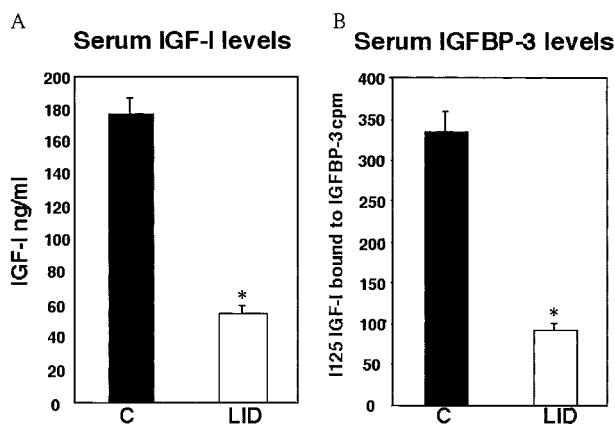
B Serum IGFBP-3 levels

Fig. 1. LID mice have lower levels of circulating IGF-I and IGFBP-3 as compared with control littermates. Blood samples were obtained at the end point of the study. IGF-I (*A*) was determined using a RIA, and the serum IGFBP-3 levels (*B*) were determined using a ligand blot assay; *bars,* ± SD.

MATERIALS AND METHODS

Animals and Treatments. LID and C littermate virgin female mice, were housed 5–6/cage in a temperature- and light-controlled room. The generation and genotyping of control and LID mice has been described previously (23, 25).

DMBA-induced Mammary Tumor Model. Virgin control and LID females (which were littermates) were fed with AIN-76 diet containing 8.4% linoleic acid (purchased from ICN, Irvine, CA) from 4 weeks of age throughout the experiment. At 6 weeks of age mice were treated intragastrically (by gavage) with 0.1 ml of corn coil containing DMBA (Sigma Chemicals, St. Louis, MO) at a dose of 1 mg/week once a week for 7 weeks. Mice were observed daily and sacrificed at 22–23 weeks of age. Blood was collected from the retro-orbital sinus before euthanasia. Body weight of the mice and dietary intake were recorded weekly until termination of the experiment. Liver and uterine weights were recorded at termination of the experiment. After the last DMBA treatment mice were palpated at weekly intervals. Tumor size was measured with a caliper.

C3(1)/SV40-LTA-induced Mammary Tumor Model. C3(1)/SV40-LTA transgenic mice were generated and bred as described previously (24). LID female mice were crossed with homozygous C3(1)/SV40-LTA transgenic male mice. F1 mice were backcrossed to LID mice (to keep only a single copy of the C3(1)/SV40-transgene). These crosses gave rise to control and LID mice that carry the C3(1)/SV40-LTA transgene and were littermates (to control for other genetic components). Mice were observed daily and sacrificed when tumors reached 1.5–2 cm in diameter.

All of the procedures were approved by the Animal Care and Use Committee, of the National Institutes of Diabetes and Digestive and Kidney Diseases, NIH.

Immunohistochemistry. Mammary tissue or tumors were isolated and fixed in 4% paraformaldehyde overnight, embedded in paraffin blocks, and sectioned at 5 μm. Primary antibodies keratin 1, 5, or 6 (Babco, Richmond, CA) at a dilution of 1:200, and β-catenin (Transduction Labs, Lexington, KY) at a dilution of 1:100, were applied to the sections. After incubation at 37°C for 1 h, the sections were washed with PBS and incubated with Texas Red-conjugated antirabbit (Alexafluor 594) and FITC-conjugated antimouse (Alexafluor 488) secondary antibodies at a dilution of 1:400 (Molecular Probes, Eugene, OR) at 37°C for 30 min. Slides were studied using a Zeiss Axioscop microscope at ×630 magnification.

Serum Levels of IGF-I and IGFBP-3. Serum IGF-I levels were measured by RIA kit (Rat/mouse IGF-I assay system provided by Dr. Albert F. Parlow at the National Hormone and Pituitary Program, Harbor-University of California Los Angeles Medical Center, Torrance, CA) with a sensitivity of 0.02 ng/ml. Serum IGFBP-3 levels were analyzed by Western-ligand blot assay using ^{125}I-hIGF-I (Amersham Life Science, Inc., Amersham Place, Buckinghamshire, England) as described before (25, 26). Signals were quantified by Fuji phosphorimaging.

RNase Protection Assay. Tissues were homogenized with a polytron homogenizer (Brinkmann Instruments, Wesbury, NY) in RNazol B reagent (Tel-Test Inc., Freindswood, TX), and total RNA was isolated according to the manufacturer's instruction. Total RNA (50 μg) was hybridized with ^{32}P-labeled c-myc, p53, cyclin D3, proliferating cell nuclear antigen, and glyceraldehyde-3-phosphate dehydrogenase riboprobes (Pharmigen, San Diego, CA) overnight at 45°C. RNase protection assays were carried out as described previously (23, 25).

RESULTS

LID mice have lower levels of circulating IGF-I and IGFBPs as compared with control littermates (Fig. 1). The LID females had normal mammary glands and normal ductal and stromal architecture (data not shown). No detectable differences in ductal extension were found between control and LID mice. Lactation was normal in these mice, as reported previously (25).

DMBA-induced Mammary Tumor Model

The Incidence of DMBA-induced Mammary Tumors Was Significantly Lower in LID Mice Than in Control Mice. As shown in Fig. 2*A,* virgin females at 4 weeks of age were fed an AIN-76 diet containing 8.4% linoleic acid throughout the experiment. Starting at 6 weeks of age, mice received a 1-mg dose of DMBA once a week for
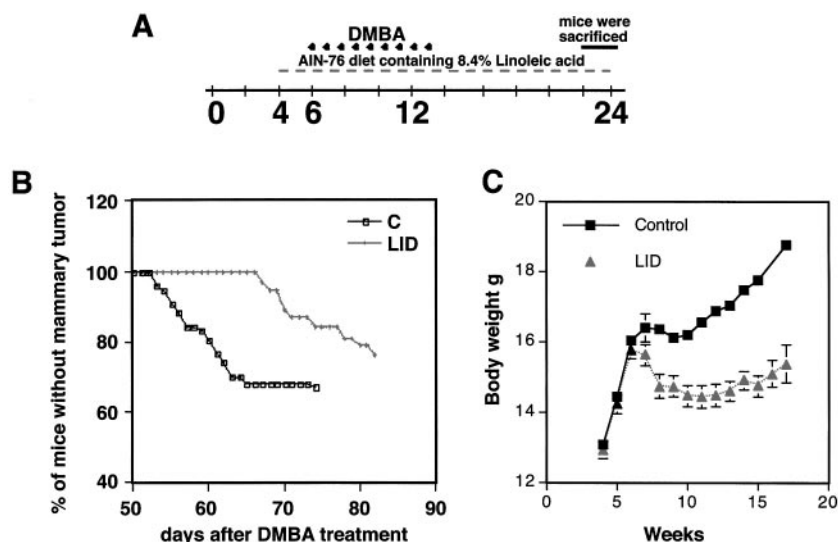
Fig. 2. DMBA-induced mammary tumors. *A,* schematic demonstration of the protocol of DMBA treatment. *B,* the onset of mammary tumors induced by DMBA is delayed in the LID mice. Mice were palpated daily, and the date a palpable tumor was observed and recorded. *C,* body weights of control and LID mice after DMBA treatment; *bars,* ±SD.

7 weeks and were sacrificed at 22–23 weeks of age. A total of 115 mice were analyzed for DMBA-induced mammary tumors. Table 1 shows that 43 of 77 (56%) control mice developed one or more mammary tumors by the age of 24 weeks. In contrast, only 10 of 38 (26%) LID mice developed mammary tumors this age. This difference in tumor incidence was highly significant ($P < 0.01$). Furthermore, the average onset of palpable mammary tumors occurred at 59.5 ± 1.1 days after cessation of DMBA treatment in control mice, whereas the average tumor onset in LID mice tumor occurred at 74 ± 1.2 days ($P < 0.01$; Fig. 2B). The tumors were mammary adenosquamous carcinomas, as reported previously with this DMBA model (27–29). The effects of DMBA treatment on food intake did not differ between control and LID mice. However, control mice gained body weight after DMBA treatment, whereas LID mice lost weight (with no evidence of vomiting; Fig. 2C). Additionally, LID mice were more susceptible to DMBA-induced toxicity than control mice, and a higher percentage of LID mice died before termination of DMBA treatment (these animals were not included in the study). At this point, we cannot exclude the possibility that the toxicity of the drug itself affected tumor incidence, although this is unlikely, because the effect of the drug is short, and we evaluated the mice 10 to 13 weeks after termination of DMBA treatment.

Metaplasia in Mammary Tumors, Induced by DMBA, Was Reduced in LID Mice. As shown in Fig. 3, extensive metaplasia was detected in late-stage mammary tumors of control mice. Several squamous cell layers and keratin deposits in the lumina were observed (Fig. 3, *panel 2*). In contrast, late-stage mammary tumors from LID mice exhibited extensive hyperplasia, but little metaplasia was detected (Fig. 3, *panels 6* and *7*). CK 5 and 6 were expressed in LID and control mammary tissue, to the same extent (Fig. 3, *panels 4, 5, 9,* and *10*). This suggests that mammary tissue underwent hyperproliferation regardless of the IGF-I levels. However, CK1 was expressed in control mice mammary tissue but not in LID mammary tissue (Fig. 3, *panels 3* and *8*). The pattern of keratin expression in tissue from control mice treated with DMBA is similar to that in epidermis. In contrast, only hyperplasia was observed in the LID mice.

Staining of mammary tumors at late stages of tumorigenesis with an anti-ki67 antibody (to detect proliferation) or terminal deoxynucleotidyl transferase-mediated nick end labeling (to detect apoptosis) did not reveal significant differences between the effects of DMBA in control and LID mice (data not shown).

C3(1)/SV40-LTA-induced Mammary Tumor Model

Mammary Tumor Onset in LID Mice Carrying the SV40 LTA Was Significantly Delayed. As shown in Fig. 4A, tumor formation occurred earlier in the control (24.1 ± 1.1 weeks) as compared with LID mice (30.17 ± 1.71 weeks) carrying the C3(1)/SV40-LTA transgene. Control mice exhibited higher numbers of tumors, with 60% of control female mice developing two or more mammary tumors, whereas only 30% of LID mice developed two or more mammary tumors (Fig. 4B). In addition, as shown in Fig. 4C, palpable tumor volume was greater in control than LID mice carrying the C3(1)/SV40-LTA. Histological examination of tumors, obtained from control and LID mice at late stages of tumorigenesis revealed no differ-
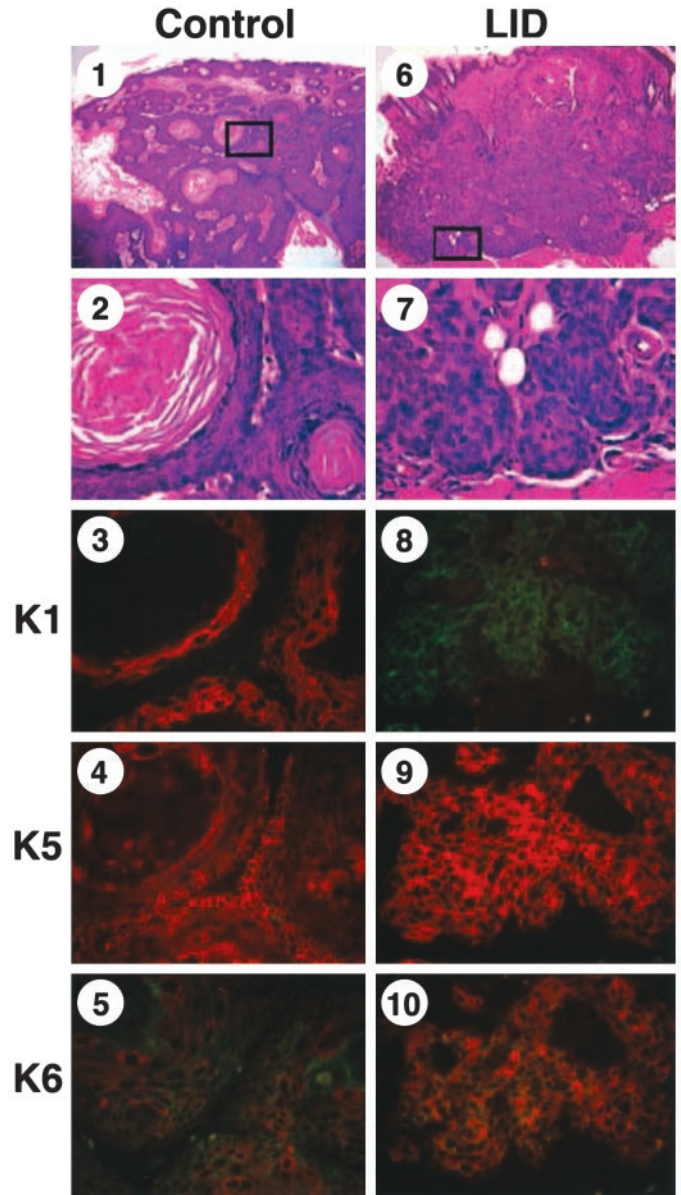

Fig. 3. DMBA-induced extensive metaplasia in mammary tissue of control mice and to a lesser extent in LID mice. *Panels 1, 2, 6,* and *7* show H&E staining. *Panels 3, 4, 5, 8, 9,* and *10* show immunohistochemistry with various keratin (*red*) and β−catenin (*green*) antibodies. Extensive metaplasia in mammary tissue of control mice at late stage is shown in *1* and in *2* with 6.3 times larger magnification of the *black square* shown in *panel 1*, mammary tissue from LID mice at late stage exhibiting extensive hyperplasia but little metaplasia with keratinization as shown in *6* and *7* with 6.3 times larger magnification of *black square* shown in *panel 6*. Keratin 5 (*K5*) and keratin 6 (*K6*) were expressed in LID mammary tissue, -9 and -10, similar to that in control mice, -4 and -5. Keratin 1 (*K1*) was expressed in control mammary tissue -3 but not in LID-8 mammary tissue.

ences between the two groups. In both control and LID mice, H&E staining shows invasive adenocarcinoma. Staining of tumors at late stage of tumorigenesis with an anti-ki67 antibody or terminal deoxynucleotidyl transferase-mediated nick end labeling staining did not reveal any differences between tumors obtained from control and LID mice carrying the C3(1)/SV40-LTA transgene (data not shown).

Expression Levels of p53, ki-ras, and the IGF-I Receptor Are Similar in Late Mammary Tumors from LID and Control Mice Carrying the C3(1)/SV40-LTA. One of the mechanisms by which C3(1)/SV40 -LTA induces cell transformation is thought to involve its ability to bind and inactivate the tumor suppressor gene *p53*. Furthermore, it has been shown that during cell transformation, there is a gain

Table 1 *Mammary tumor incidence after DMBA treatment*

Group	n	Mice displaying mammary tumor (%)	Onset of palpable tumor[a]
Control-DMBA	77	43 (55.8)[b]	59.5 ± 1.1[c]
LID-DMBA	38	10 (26.3)	74 ± 1.2

[a] Days after cessation of DMBA.
[b] Control-DMBA versus LID-DMBA, $P < 0.01$.
[c] Control-DMBA versus LID-DMBA, $P < 0.01$.

Fig. 4. SV40-LTA-induced mammary tumors. *A,* the onset of mammary tumors induced by SV40-LTA is delayed in the LID mice. Mice were palpated daily, and the date a palpable tumor was observed and recorded. *B,* the majority of LID mice developed one mammary tumor per mouse, whereas the majority of control mice developed two or more mammary tumors. *C,* tumor volume was larger in control mice as compared with LID mice. *D,* RNase protection assay of the IGF-IR, p53, and ki-ras demonstrate that there is an increase of those mRNAs in both control and LID mice carrying the SV40-LTA transgene compared with normal mammary tissue (*N*). There was no difference in gene expression of the IGF-I R, p53, and ki-ras between control and LID mice carrying the SV40-LTA transgene.
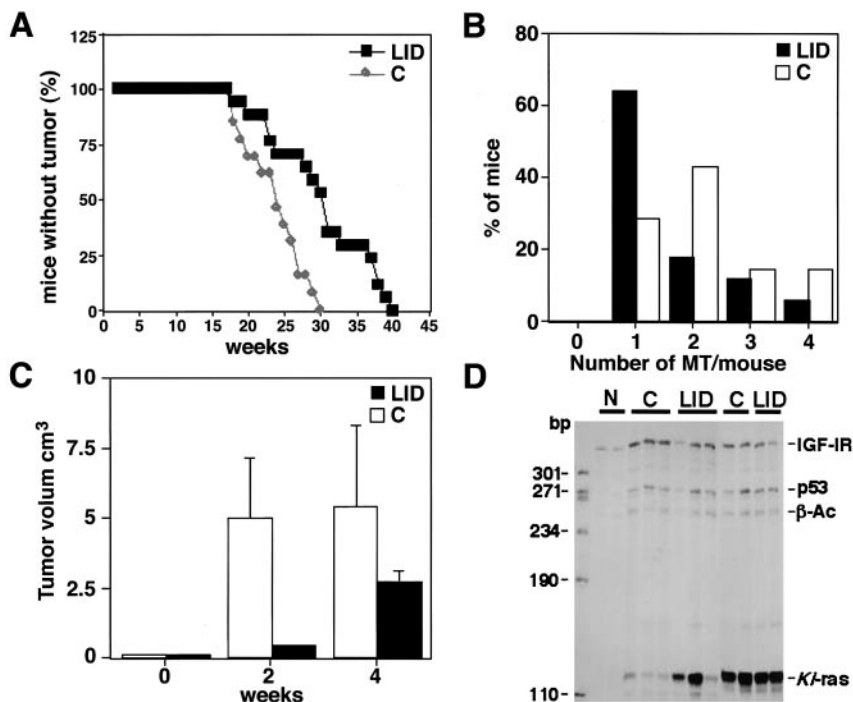
of genomic material from the distal region of chromosome 6, which encodes the transforming protein ki-ras. To determine expression levels of p53 and ki-ras, carcinomas were isolated from control and LID mice, and RNase protection assays were performed. Signals were corrected for β-actin protected bands. As shown in Fig. 4D, expression of p53 and ki-ras mRNA levels did not differ between control and LID mice. Furthermore, there were no differences in the expression of the IGF-IR in mammary tumors obtained from control and LID mice.

DISCUSSION

Rodent models of chemically or genetically induced mammary tumors have been used for years to obtain insight into the biology of breast cancer. The mechanism by which chemical carcinogenesis induces mammary tumors in rodents is not well understood. The carcinogen is believed to bind to DNA and cause a mutational event, referred to as initiation, whereas systemic factors, including hormones and other growth-enhancing factors, are responsible for the promotion phase of tumorigenesis. The latter is characterized by transformation of preneoplastic cells to neoplastic tumor cells, a process that requires cell proliferation.

It has been shown that energy-restricted diets, which significantly reduce levels of circulating IGF-I, decrease the incidence of cancer in rodents (30, 31). In addition, experiments with *lit/lit* mice, which have a mutation in the growth hormone releasing hormone receptor and as a result have only 10% of normal circulating growth hormone and IGF-I levels, showed that neoplastic proliferation of human MCF-7 cell xenografts is significantly attenuated in these mice (32). Transgenic mice that overexpress growth hormone, and consequently have high levels of circulating IGF-I, exhibit morphological evidence of mammary gland epithelial cell hyperplasia and a high frequency of breast cancer (33, 34). In contrast, transgenic mice overexpressing a growth hormone antagonist display ductal hypoplasia (35).

We examined tumor incidence in LID mice and control littermates that were subjected to either chemically or genetically induced mammary tumors, to determine whether circulating IGF-I levels can influence breast carcinogenesis. Here we show that reduced levels of

circulating IGF-I were associated with an increased latency of tumor formation and reduced tumor size in both DMBA- and C3(1)/SV40-LTA-induced mammary tumor models. Furthermore, there was a decrease in the incidence of tumor formation specifically in the DMBA model.

DMBA treatment typically induces mammary tumors in >50% of virgin mice by the age of 1 year. In addition to their delayed appearance, DMBA-induced mammary tumors in LID mice exhibited distinct morphological appearances. Specifically, whereas virtually all of the tumors in control mice displayed extensive squamous metaplasia, this was rarely seen in tumors from LID mice. It could be argued that squamous metaplasia is an end point of DMBA-induced tumors, and its absence in LID mice results from delayed tumor progression. However, we also compared tumors of the same size from control and LID mice and observed squamous metaplasia almost exclusively in the control mice. This suggests that the level of circulating IGF-I determines the type of tumor induced by DMBA. Whereas low levels of IGF-I preferentially induce adenocarcinomas, high levels induce squamous metaplasia. In their studies, Satyamoorthy *et al.* (36) detected different expression patterns of CK-1, -5, and -6, depending on the stage of tumor formation in mice. To determine the state of metaplasia, we used various types of anti-CK antibodies to analyze the tumors observed in LID and control mice. Whereas mammary tissue of control mice expressed high levels of CK1 in the middle layers of the tumors, mammary tissue from LID mice did not express increased levels of CK1. However, mammary tissues from both control and LID mice expressed high levels of both CK6 and CK5. Taken together, it appears that lower levels of circulating IGF-I can repress metaplasia but still cause hyperplasia.

The SV40-LTA has been used as a transforming agent in numerous cell types, both *in vivo* and *in vitro*. The C3(1)/SV40-LTA transgene was found to be expressed primarily in the distal mammary ductal epithelium and in terminal ductal lobular units, which leads to the progressive development of mammary cancers (24). Crossing C3(1)/SV40-LTA transgenic mice with LID mice resulted in mice with reduced circulating levels of IGF-I and a "genetic predisposition" for

mammary tumor formation. These mice were compared with control (C3(1)/SV40-LTA) mice, which have normal levels of IGF-I and the same "genetic predisposition." Our observations show that low levels of circulating IGF-I in the LID mice delayed tumor formation. Moreover, most of the control mice developed more than one mammary tumor per mouse, whereas LID mice usually developed only one mammary tumor per mouse.

One of the mechanisms of C3(1)SV40-LTA-induced tumorigenesis involves the binding of this transforming protein to the p53 tumor suppressor gene product and the retinoblastoma protein (37, 38). This leads to functional inactivation of p53 and retinoblastoma, and impairs normal regulation of the cell cycle, thereby increasing cell proliferation and blocking normal apoptotic responses. Here, we show that p53 levels were higher in mammary tumor tissue than in normal mammary tumor. However, we could not find any difference between mammary tumors obtained from control and LID mice. This might be explained by the fact that the tumors were analyzed at relatively late stage of development. We also observed an increase the levels of ki-ras, a transforming factor, in both control and LID mice carrying the C3(1)/SV40-LTA transgene. This increase most likely results from accumulation of genomic material from chromosome 6, which encodes the ki-ras, as was demonstrated in previous studies (39). Thus, the effect of reduced circulating levels of IGF-I on tumor growth does not appear to be mediated via these tumor gene products, suggesting that IGF-I may directly influence cellular proliferation by activating the IGF-IR on malignant breast cells.

In summary, we show additional evidence that circulating IGF-I is involved in the pathophysiology of neoplasia, both in terms of the risk of cancer developing in a predisposed state and the behavior of cancers.

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